AFRL-RX-WP-JA-2014-0200

A DIRECT QUANTITATIVE AGAR-PLATE BASED ASSAY FOR ANALYSIS OF PSEUDOMONAS PROTEGENS PF-5 DEGRADATION OF POLYURETHANE FILMS (POSTPRINT)

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AFRL/RXAS

OCTOBER 2014
Interim Report

AIR FORCE RESEARCH LABORATORY
MATERIALS AND MANUFACTURING DIRECTORATE
WRIGHT-PATTERSON AIR FORCE BASE, OH 45433-7750
AIR FORCE MATERIEL COMMAND
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED *(From – To)*
October 2014	Interim	14 September 2009 – 13 September 2014

4. TITLE AND SUBTITLE
A DIRECT QUANTITATIVE AGAR-PLATE BASED ASSAY FOR ANALYSIS OF PSEUDOMONAS PROTEGENS PF-5 DEGRADATION OF POLYURETHANE FILMS (POSTPRINT)

5a. CONTRACT NUMBER
FA8650-09-D-5037-0003

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
61102F

6. AUTHOR(S)
(see back)

5d. PROJECT NUMBER
3002

5e. TASK NUMBER

5f. WORK UNIT NUMBER
X06W

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
(see back)

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Air Force Research Laboratory
Materials and Manufacturing Directorate
Wright Patterson Air Force Base, OH 45433-7750
Air Force Materiel Command
United States Air Force

10. SPONSOR/MONITOR'S ACRONYM(S)
AFRL/RXAS

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
AFRL-RX-WP-JA-2014-0200

12. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution A. Approved for public release; distribution unlimited. This report contains color.

13. SUPPLEMENTARY NOTES
PA Case Number: 88ABW-2014-1722; Clearance Date: 14 April 2014. Journal article published in International Biodeterioration & Biodegradation 95 (2014) 311-319. The U.S. Government is joint author of the work and has the right to use, modify, reproduce, release, perform, display or disclose the work. The final publication is available at http://dx.doi.org/10.1016/j.ibiod.2014.09.005.

14. ABSTRACT
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15. SUBJECT TERMS
pseudomonas biofilms, polyurethane, biodegradation, FTIR spectroscopy, citrate, impranil

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON *(Monitor)*
a. REPORT	b. ABSTRACT	c. THIS PAGE			Wendy J. Goodson
Unclassified	Unclassified	Unclassified	SAR	13	19b. TELEPHONE NUBER *(include area code)* (937) 255-9385

REPORT DOCUMENTATION PAGE Cont'd

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International Biodeterioration & Biodegradation 95 (2014) 311–319

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

A direct quantitative agar-plate based assay for analysis of *Pseudomonas protegens* Pf-5 degradation of polyurethane films

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ARTICLE INFO

Article history:
Received 1 August 2014
Received in revised form
11 September 2014
Accepted 13 September 2014
Available online 2 October 2014

Keywords:
Pseudomonas biofilms
Polyurethane
Biodegradation
FTIR spectroscopy
Citrate
Impranil

ABSTRACT

A quantitative assay was developed for the direct measurement of polymer film degradation from bacterial colonies on agar plates. Small (1 mm diameter) colonies of *Pseudomonas protegens* Pf-5 (formerly *Pseudomonas fluorescens* Pf-5) were used for this work. Interactions between the Pf-5 colonies and thin polyurethane (PU) coatings on ZnSe coupons were evaluated for degradation using infrared spectroscopy. Three different coatings were analyzed and were formed from 1) a colloidal, aqueous – based polyester PU (Impranil® DLN); 2) an organic solvent – based polyester PU (Irogran); and 3) an organic solvent – based polyether PU (AS-P108). Over a 24 h time period at 30 °C, citrate exposed Pf-5 cultures rapidly degraded Impranil coatings, consistent with analogous zone clearing assays. However, the Irogran and AS-P108 PU's, which are not directly compatible with zone clearing assays, showed no measureable degradation by the coating assay under identical conditions. These results demonstrated the capability to evaluate any variety of polymer formulation as solid films under identical biological conditions. The results also show that rapid microbial degradation of colloidal polyurethanes such as Impranil are not necessarily representative of activity towards other PU materials.

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1. Introduction

The wide use of polyurethanes (PU) in our society makes their biodegradation of equal importance as their manufacturing (Zia et al., 2007). The balance between creating stable polymers that resist degradation and minimize their potential long-term environmental impact continues to be one of the major issues with the general use of these materials. Straightforward screening assays are required to analyze complex relationships between degradation of synthetic polymers and microbial physiology (Mukherjee et al., 2011). Qualitative assays have been developed to systematically assess biological degradation of PU using agar supports containing a slurry of colloidal PU (Impranil). The appearance of clearing zones around large ~1 cm diameter bacterial colonies is indicative of degradation (Ruiz and Howard, 1999). Examples of the results from this type of assay using *Pseudomonas protegens* Pf-5 cultures are shown in Fig. 1. While these assays are useful for a *qualitative* screening of microorganisms, they have substantial drawbacks. First, polyurethanes are rarely used in their colloidal form but rather as coatings and other solid forms for applications. Second, zone clearing assays do not assess the processes that may occur at the polymer coating surface and cannot be performed with polymer films. Finally, since zone clearing assays rely heavily on diffusion of degradative proteins or compounds into the agar, they have limited sensitivity, and lack the ability to detect subtle differences in degradation due to colony age and size. Instead of relying on the diffusion of proteins into a solid support containing colloidal polymers this work describes an assay for polymer films with transmission micro-Fourier transform infrared (μFT-IR) analysis to sensitively and quantitatively assess degradation of PU polymers using bacterial colonies <24 hrs into formation.

Pseudomonas spp. are γ-proteobacteria commonly found in water or soil and plant rhizospheres (Lapouge et al., 2007; Takeuchi et al., 2012) that can metabolize citrate for the degradation of nitrates and aromatic compounds (Elefsiniotis et al., 2004; Yang et al., 2012). Citrate has been shown to be a carbon source that allows for

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http://dx.doi.org/10.1016/j.ibiod.2014.09.005
0964-8305/Published by Elsevier Ltd.

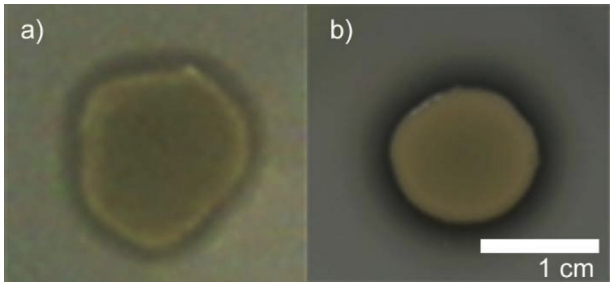

Fig. 1. Impranil infused zone-of-clearing assay using Pf-5 cultures grown on M9 supplemented with (a) 10 mM citrate or (b) LB media. Clearing zones can be observed around the cultures, indicating Impranil degradation.

growth in minimal defined media of *P. protegens Pf-5* (formerly called *Pseudomonas fluorescens* Pf-5) and leads to variations in proteinase activity, which can be linked to esterase activity (Nicolaisen et al., 2012). Several polyurethane degrading enzymes have been identified from *P. fluorescens* (Loredo-Trevino et al., 2012; Shah et al., 2013) and *Pseudomonas chlororaphis* (Howard et al., 2001a; Howard et al., 2007) using Impranil in planktonic culture or in zone clearing assays for cultures on solid media. In *P. chlororaphis*, the predominant polyurethane degrading enzymes are polyurethane esterases A and B (*Pue*A and *Pue*B, respectively), which have also been identified in the Pf-5 genome (Howard et al., 2007).

The objective of this work was to design a sensitive, quantitative PU degradation assay that allows for the direct measurement of *polymer film* degradation caused by small bacterial colonies on agar plates. This agar-based plate assay consists of exposure of PU coatings on a ZnSe substrate to <1 mm diameter colonies of *P. protegens* Pf-5. Our approach was to employ optically transparent coupons as substrata so that the coating materials could be probed directly by optical microscopies following exposure to cultures while limiting diffusion of polymer degradation products from the surface. Thus, unlike zone clearing assays, this assay does not require the secretion of active PU degrading components 1 mm–10 mm from the colony. In this study, standardized procedures were developed for agar plate-based cultures of *P. protegens* Pf-5, grown on M9 and Luria–Bertani (LB) media, for 24 h degradation assays of PU coatings. The advantage of this flexible approach is that it enables the evaluation of any variety of polymer formulation under controlled biological conditions.

2. Materials and methods

2.1. Microbial strains and chemicals

P. fluorescens Pf-5 (ATCC BAA-477) and *Escherichia coli* K-12 were stored at −80 °C in stocks of 50% glycerol and were reconstituted on LB agar. M9 minimal medium (Sambrook and Russell, 2001) was used as the agar-based support for all experiments except when noted differently in the text. Sodium citrate (Sigma–Aldrich), agar (Fisher), and M9 salts were used as received. ZnSe optical windows (1" dia. × 0.08" thick) coupons were purchased from Phoenix Infrared Corp. Impranil® DLN, an anionic polyester polyurethane (PU) colloidal dispersion, was provided by Bayer Material Science. Irogran, also a polyester PU, was obtained from Huntsman (Auburn Hills, MI) in pellet form. AS-P108 polyether PU resin and catalyst were obtained from CAAP Co, Inc (Milford, CT). The resin had the carbon black antistatic additive removed via centrifugation (Crookes-Goodson et al., 2013).

2.2. 2.2. Growth conditions of P. protegens Pf-5 in liquid culture

Planktonic cultures were initiated by inoculation of medium with a single colony of Pf-5. Cultures were grown in M9 medium containing 10 mM citrate at 30 °C with orbital shaking at 100 rpm. Absorbance at 600 nm (OD_{600nm}) was used to monitor the cell density. Additional information regarding the growth characteristics and culture considerations for *P. protegens* Pf-5 is provided in the supplemental information.

2.3. Coating of ZnSe coupons with a polymer

ZnSe coupons were cleaned by heated sonication in detergent and DI water for 1 h, and 3 × 15 min sonicated rinses in DI water followed by a final sonicated rinse in 100% ethanol. Coupons were dried in a sterile hood. Once dried, the coupons were treated with an oxygen plasma for 10 min at 33 Pa and 130 W. Within 10 min after removal from the plasma oven chamber, the coupons were placed in a sterile hood where the polymer coatings were applied. Each polymer was applied from a solution: 3% v/v Impranil was dissolved in methanol; Irogran (1 g) was dissolved in 25 mL THF; and AS-P108 was prepared by adding 0.3 g of catalyst to 10 g of resin and then diluted with 10 mL of PUT-10 PU thinner (Caapco). Coupons were coated with 8–10 droplets (~420 μL) of the respective polymer in solution, until the coupon was uniformly wetted. Impranil and Irogran solutions were dried for at least 2 h and AS-P108 was cured for at least 3 days. The resulting Impranil, Irogran, and AS-P108 coatings were approximately 2 ± 1 μm thick. The thicknesses of the coatings were determined from interference fringes in FTIR spectra of the coatings (Griffiths and De Haseth, 2007).

2.4. Plate assay

The agar plates used for these experiments were poured from a sterilized (121 °C, 15 min) solution of 7.5 g agar (granules) in 500 mL of M9 salt solution containing 3 mM $MgSO_4$, 200 μM $CaCl_2$ and 10 mM potassium citrate (below 50 °C). To identify localized pH gradients around colonies over time, 300 μL of a 15 mg/L unbuffered sterile solution of phenol red (Sigma) was added to the above-mentioned media. Overnight Pf-5 cultures were diluted to 1 × 10^5 colony forming units (cfu) per ml to control the size of the cultures to be assayed. Dilutions of Pf-5 planktonic cultures were spotted on the agar plates to avoid the formation of large multiclonal colonies (>1 cm diameter). The culture dilutions (1 × 10^5 to 10^7 cfu/mL) were then drop-cast (10 μL each) in six separate spots on each plate and the plates were incubated at 30 °C for at least 24 h to establish monoclonal bacterial growth on the plate.

Polymer degradation assays were started by placing polymer-coated ZnSe coupons on top of clusters of individual (<1 mm radius) colonies. The general schematic for this assay is illustrated in Fig. 2. After 24 h of exposure, coupons were removed and analyzed in triplicate by FT-IR with the coating side up. Detailed analysis was performed within regions near the culture edge. Two control experiments were performed in parallel with the Pf-5 degradation experiments. The first control was Impranil coated coupons placed on sterile agar plates. For the second control experiment, Impranil coated coupons were placed on <1 mm diameter colonies of *E. coli* to confirm that general colony formation does not compromise coating integrity. LIVE/DEAD Baclight (Life Technologies) staining of the bacteria using the manufacturer's protocol for microscopy in the assay confirmed that the cells were still viable at the end of all experiments (Supplemental material). All experiments were performed in triplicate from three independent biological replicates.

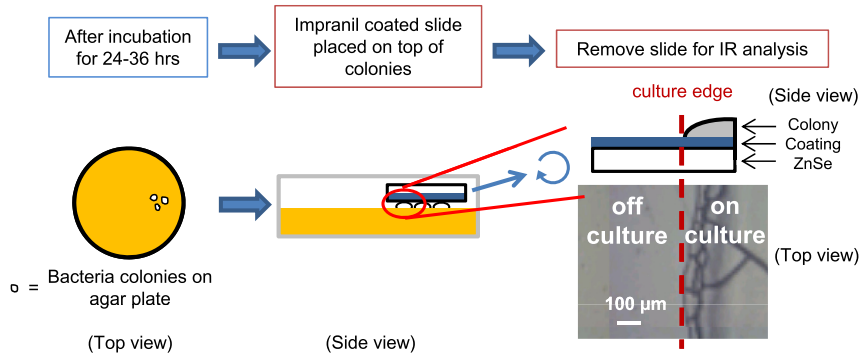

Fig. 2. General schematic of plate based experiments used to determine PU degradation.

2.5. IR measurements and AFM images

μFTIR spectra were acquired with a Thermo Scientific Continuum microscope/6700 FTIR spectrometer using liquid-nitrogen cooled mercury cadmium telluride (MCT) detectors with either 50 μm or 250 μm detector element sizes (for IR apertures <50 μm or >50 μm, respectively). All spectra were acquired at 4 cm^{-1} resolution. Both infrared and optical images were acquired with this microscope using a 15× Cassegrain objective. Coatings were analyzed before and after culture exposure relative to two fiducial points allowing the same areas on the coatings to be compared before and after culture exposure. Large area IR maps (~10 × 10 mm^2) were acquired of coatings prior to culture exposure with a 150 μm microscope aperture. IR maps following culture contact were acquired with a 35 μm microscope aperture and encompassed areas of ~0.2–15 mm^2 in regions where cultures had contacted the coatings. Portions of the cultures generally remained adhered to the coatings after removal of the coated coupons from the agar plate and spectra were acquired on and off the culture regions. Removal of the culture from the coated coupons was attempted using either sonication, swabbing, or dipping of coupons in ethanol, but typically the coating material was removed as well, particularly when Impranil was used. Therefore, IR coating analysis was performed with the culture in place. Tapping mode atomic force microscopy (AFM) images were acquired with a Veeco Dimension AFM and Bruker Nanoscope 5 controller using Bruker TESP-7 probes. FTIR and AFM analyses were also performed on cultures grown on uncoated coupons, unexposed coatings, and coatings exposed to sterile plates. Selected results are in Supplemental Information.

3. Results

3.1. General growth characteristics of Pf-5 on M9/citrate agar plates

Dilutions of *P. protegens* Pf-5 cultures (grown in M9/10 mM citrate) were drop-cast onto M9/agar plates to probe the role of nutrients on PU degradation while keeping overall colony sizes <1 mm in diameter. Since this assay offers an approach to probe microbial coating degradation as a function of medium and carbon source, steps were also taken to ensure that culture growth was maintained only by plate nutrients. For this aspect, it was found that maintaining small colony sizes through dilution was imperative since drop-casting 10 μL of undiluted Pf-5 cultures directly onto M9 plates (with no carbon source) could support growth while no growth was observed with diluted cultures (data not shown). This also shows that Pf-5 can sustain growth through complex culture dynamics at high cell concentrations. Culture dilutions of <1 × 10^5 were spotted six times on the agar plate and plates were incubated for 24 hr at 30 °C. Overall growth was documented photographically making it straightforward to assess colony dimensions from properly scaled images.

To identify optimal conditions for this assay, the growth of Pf-5 colonies was investigated as a function of citrate concentration in the agar plates. Three concentrations of citrate were tested: 10 mM, 20 mM, and 60 mM (pH 7.2) in M9 media (Fig. 3). Citrate concentrations above 20 mM thwarted growth of the diluted cultures on the agar plates. Similar trends were also observed when undiluted liquid cultures were streaked directly onto the same plate formulations (Supplemental Material). The fact that colony sizes formed on 10 or 20 mM citrate were essentially equivalent indicated that either concentration was a suitable for this assay.

The growth of Pf-5 in liquid culture, changes in citrate concentration, and pH were monitored to determine the state of the cells that were drop-cast on the plates and to keep the inoculum consistent among assays (Supplemental Material). Planktonic growth in M9/citrate was linked to a decrease in acidity of the growth medium. This acidity change can impact not only cell physiology but also the activity of putative exoenzymes involved in degradation (Howard et al., 2012). Thus, the pH of agar adjacent to cultures was also assessed, using a colorimetric pH sensitive indicator (phenol red) within the agar plates (Fig. 4a and b). The development of red color near the cultures indicated that the local pH reached >8.5 (Oh et al., 2002). Minimal color change was observed over a 48 h period for small (~1 mm diameter) colonies resulting from drop-casting diluted cultures (Fig. 4a) but substantial color change was observed after 48 h using larger (1 cm diameter) colonies (Fig. 4b). Note that these cultures were not exposed to PU, so the pH changes were not a result of PU degradation. No substantial differences in colony size or morphology were observed from colonies that were exposed to phenol red

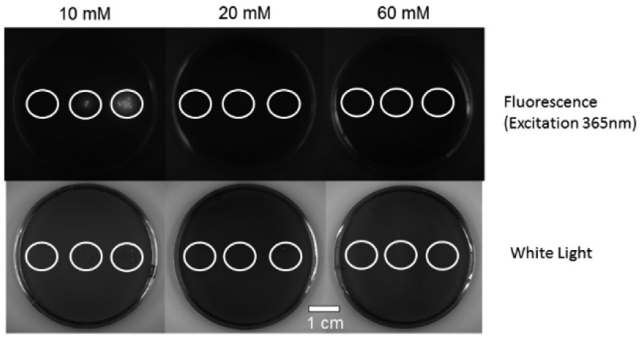

Fig. 3. Images of Pf-5 cultures diluted to 10^5–10^7 cfu/ml (left to right) and plated on 10 mM (left panels), 20 mM (middle panels), and 60 mM (right panels) citrate/M9 agar plates. White circles indicate where cultures were drop-cast on plate.

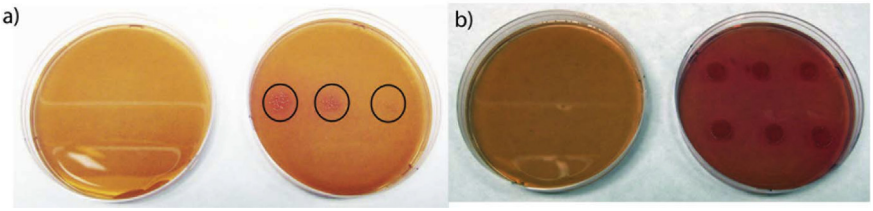

Fig. 4. Images of Pf-5 cultures grown on M9/10 mM citrate agar plates with 0.1% phenol red indicator for 48 h. (a) Small colonies resulting from drop-casting dilutions of 10^5–10^7 cfu/mL (right to left); (b) Large colonies resulting from drop-casting undiluted Pf-5 cultures (Plate on left is unexposed control plate and did not show any growth). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compared to assay agar plates making direct comparisons between polymer degradation and acidity possible. The negligible change in agar pH observed adjacent to small vs. large colonies provided further justification for using smaller cultures for the subsequent μFT-IR-based assay, helping to ensure better experimental control over the microbe state and plate conditions.

3.2. Covered versus uncovered bacterial colonies on agar plates

In order to observe culture-polymer interactions using optical and infrared spectroscopic measurements, inverted solid ZnSe windows were placed on top of growing Pf-5 colonies (Fig. 2). McCarthy et al. (1997) have previously discussed transmission μFT-IR for quantitative analysis of polyurethane degradation and applied the method to analyze microtome sections from subcutaneous PU implants (McCarthy et al., 1997). Because it is possible that covering the cultures with ZnSe windows could alter bacterial physiology, the effects of covering the bacterial films were monitored by assessing pH, differences in colony morphology using top down fluorescence microscopy, and viability of the cells with live-dead staining of the cultures. While the covered colonies caused significantly less change in the phenol red plates than uncovered colonies, subsequent LIVE/DEAD staining of cultures and imaging by confocal microscopy showed that both covered (Supplemental Fig. S4) and uncovered (not shown) colonies were viable. Native fluorescence from the uncovered Pf-5 colonies (excitation at 365 nm) enabled determination of the typical colony size when covered (Fig. 5).

3.3. Polyurethane degradation assay

Typical results from zone clearing assays with Pf-5 colonies on M9 or LB agar infused with colloidal Impranil are shown in Fig. 1. Clearing zones were observed around each of the large 1 cm colonies. In order to move beyond this qualitative observational analysis to a more quantitative spectroscopic analysis, we developed an agar-plate based assay in which an Impranil coated ZnSe coupon was placed in contact with small Pf-5 colonies grown on M9 media (Imp-Pf5-M9) shown in Fig. 6. As indicated in Fig. 6, analysis focused on the edge of the colony where biomass was minimal. An IR map with the color scale corresponding to the intensity of the 1735 cm^{-1} carbonyl absorbance from the urethane and ester components is shown in Fig. 6a. The culture covered PU coating is located in the lower half of the image and the upper half is bare PU. The boxed region identifies an area containing the culture edge, identified in the optical micrograph in Fig. 6b. Note that the color change in Fig. 6b is due to the diffraction of visible light and not representative of chemical mapping. The dashed line marks the culture edge; the culture is located below the line. The lower portion of the optical image shows that the culture has a cracked appearance. These cracks appear to be in the culture, not the coating, and begin appearing within minutes after the coupon was removed from the plate. The culture region just in proximity to the dashed line is very thin and is possibly new bacteria growth that occurred after the coupons were placed over the culture. Fig. 6c shows an AFM image from this region verifying the presence of monolayer clusters of bacteria.

Examples of individual IR spectra are shown in Fig. 6d and were acquired at the corresponding numbered points in Fig. 6a and b. Spectra 1 and 2 were acquired off the culture, about 2000 μm and 500 μm from the culture edge, respectively. Spectra 3 and 4 were acquired on the culture about 200 and 800 μm from the edge. Spectrum 1 remains similar to the unexposed coating spectrum (Fig. 7a and supplemental information Fig. S6). The most prominent peak in spectrum 1 is the 1735 cm^{-1} carbonyl peak used for the IR intensity map and is in a region with minor contribution from the culture. Therefore, monitoring this peak serves as a useful method for identifying coating changes. The peak intensity is highest at position 1, the furthest from the culture edge, and steadily decreases with proximity to the culture. This reduction in carbonyl peak intensity relative to the culture position can be observed in spectra 2–4 and in the IR map. A plot of the carbonyl absorbance versus position along the solid line in the IR map is also shown in Fig. 6e. For guidance, the numbered positions and culture edge in the IR map are also indicated in the plot.

In comparison with spectrum 1 in Fig. 6d, spectrum 2 shows a similar spectral profile but with decreases in all other peak intensities in addition to the 1735 cm^{-1} carbonyl peak. These include

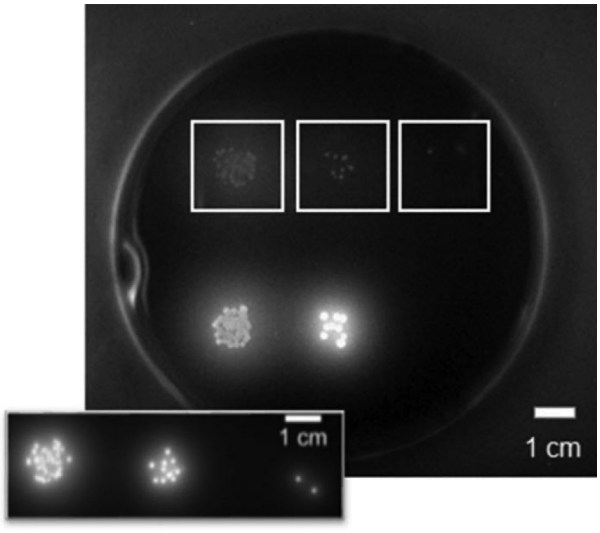

Fig. 5. Native fluorescence image of Pf-5 (Excitation at 365 nm) cultures on an M9/10 mM citrate agar plate. Cultures were formed by drop-casting dilutions of 10^5–10^7 cfu/ml (right to left) onto the plates. White rectangles represent colonies covered with glass slides. Inset: Enhanced native fluorescence image of 10^5–10^7 (right to left) dilutions drop cast with growth after 48 h.

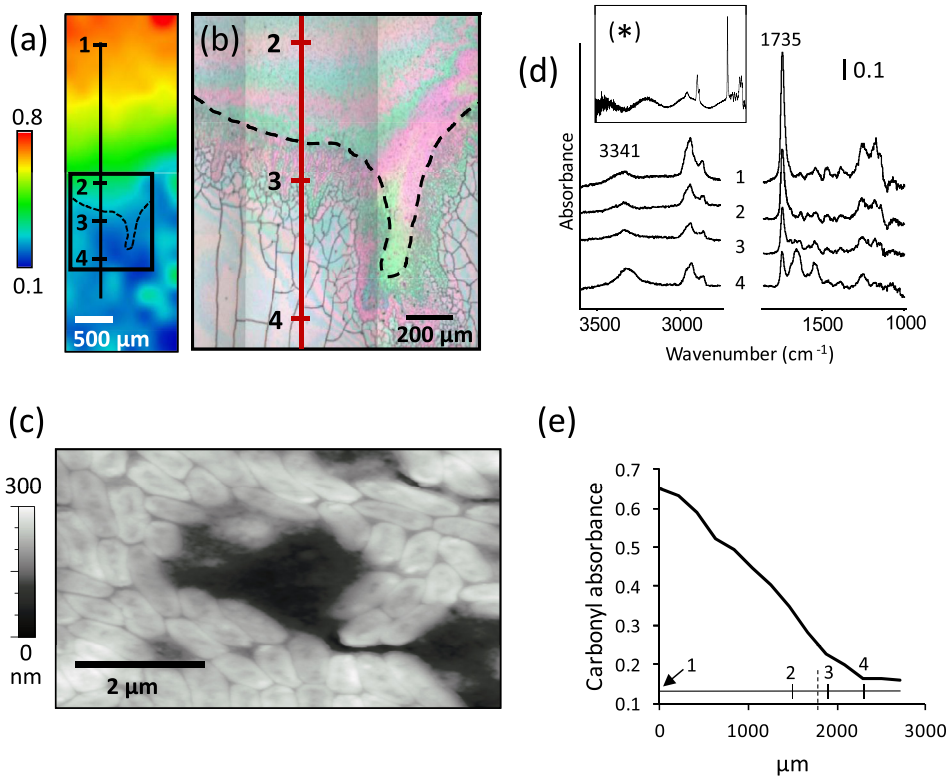

Fig. 6. FTIR and AFM analysis of an Impranil coated ZnSe coupons (Imp-Pf5-M9) (a) FTIR map of a region containing a culture edge. The color scale represents the absorbance intensity at 1735 cm^{-1}. (b) Optical micrograph of the boxed region in the IR map. The dashed line identifies the culture edge. Color in this panel is due to optical diffraction and is not related to chemical mapping. (c) AFM image from the culture edge identifying the presence of monolayer clusters of bacteria. (d) Individual IR spectra acquired at the corresponding numbered points in the IR map and optical micrograph. The inset (*) shows spectrum 1 from 7000 to 1000 cm^{-1} to reveal the interference fringes used to determine sample thickness. (e) Plot of 1735 cm^{-1} absorbance intensity along the solid line in the IR map. The numbered annotations and dashed line correspond to the numbered positions and culture edge in the map. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the N–H stretch centered near 3341 cm^{-1}, C–H stretches between 2800 and 3000 cm^{-1}, and C–O stretches in the region near 1200 cm^{-1}. However, slight variations in relative peak intensities do occur with proximity to the culture. For example, the ratios of the NH stretch (~3341 cm^{-1}) to carbonyl stretch (1735 cm^{-1}) peak areas are about 0.28 and 0.35 for spectra 1 and 2, respectively. A slight change in the relative intensities of the 1245 $cm^{-1}$and 1174 cm^{-1}peaks is also observable. Otherwise, no additional features such as new peaks or shoulders appear to emerge in spectrum 2. Only in spectrum 4 do new absorptions become clearly visible, which are attributed to the culture. These include the amide I and II peaks appearing at 1650 and 1540 cm^{-1}, respectively, and increased absorbance around 3300 cm^{-1} (N–H, O–H stretch). Spectrum 3 (culture edge) also contains a small amide I absorption (observable on close inspection), indicating low culture concentration. Additional details of the polymer coating and culture spectral features identified from individual PU and culture components are summarized in the supplemental material. FTIR spectra of coatings exposed to agar plates (not shown) showed no changes relative to the unexposed coatings.

The inset in Fig. 6d, labeled with the (*), shows spectrum 1 with an expanded range from 7000 to 1000 cm^{-1}. This expanded spectral range reveals sinusoidal oscillations characteristic of interference fringes often observed for thin samples with parallel surfaces (Griffiths and De Haseth, 2007). The sample thickness can be determined from the period of these oscillations, given by $t = 1/(2\ nT)$, where n is the index of refraction and T is the oscillation period. Using $n = 1.5$ (verified from IR reflectance measurements) expanded spectral ranges allowed thicknesses of 2.3 µm, 1.2 µm, 1.0 µm, and 1.5 µm to be calculated for spectral acquisition points 1–4, respectively. Thus, the trend in thickness tracks with carbonyl peak intensity at points 1–3, but diverges at point 4 where the culture is thicker. The divergence is attributed to the thickness measurement including both the coating and culture. The ratio for carbonyl absorbance to coating thickness for unexposed Impranil was 0.357 ± 0.003 absorbance units/µm under ambient conditions (21 °C, 45% relative humidity). Note that this value will vary with hydration and swelling of the coating, although the transmission IR absorption intensities of the polymer should not be significantly impacted.

Biodegradation of coatings can be revealed through a number of different characteristics including varying chemical and mechanical changes as well as cracking and pitting of the coating. So approaches to make quantitative comparisons of coating degradation will have some dependence on accounting for the types of degradation features observed. For the current case, Fig. 6 indicates uniform coating loss with distance from the culture edge indicating degradation that is consistent with diffusion of PU degrading agents from the culture colony; consistent with the traditional zone clearing assays. Other coating/culture/media combinations assayed either showed similar uniform material loss or negligible changes in µFTIR results. Thus, transmission µFTIR absorbance measurements on the coating within 300 µm of culture edges were used to obtain quantitative measures of polymer coating loss. As with Fig. 6, we chose to use the carbonyl absorbance intensity for quantitative assays. Given the linearity of absorbance with chemical concentration and film thickness (Beer's law), changes in the carbonyl absorbance intensity are proportional to changes in moles of carbonyl moieties per unit area of coating. Expected degradation products also appear to be

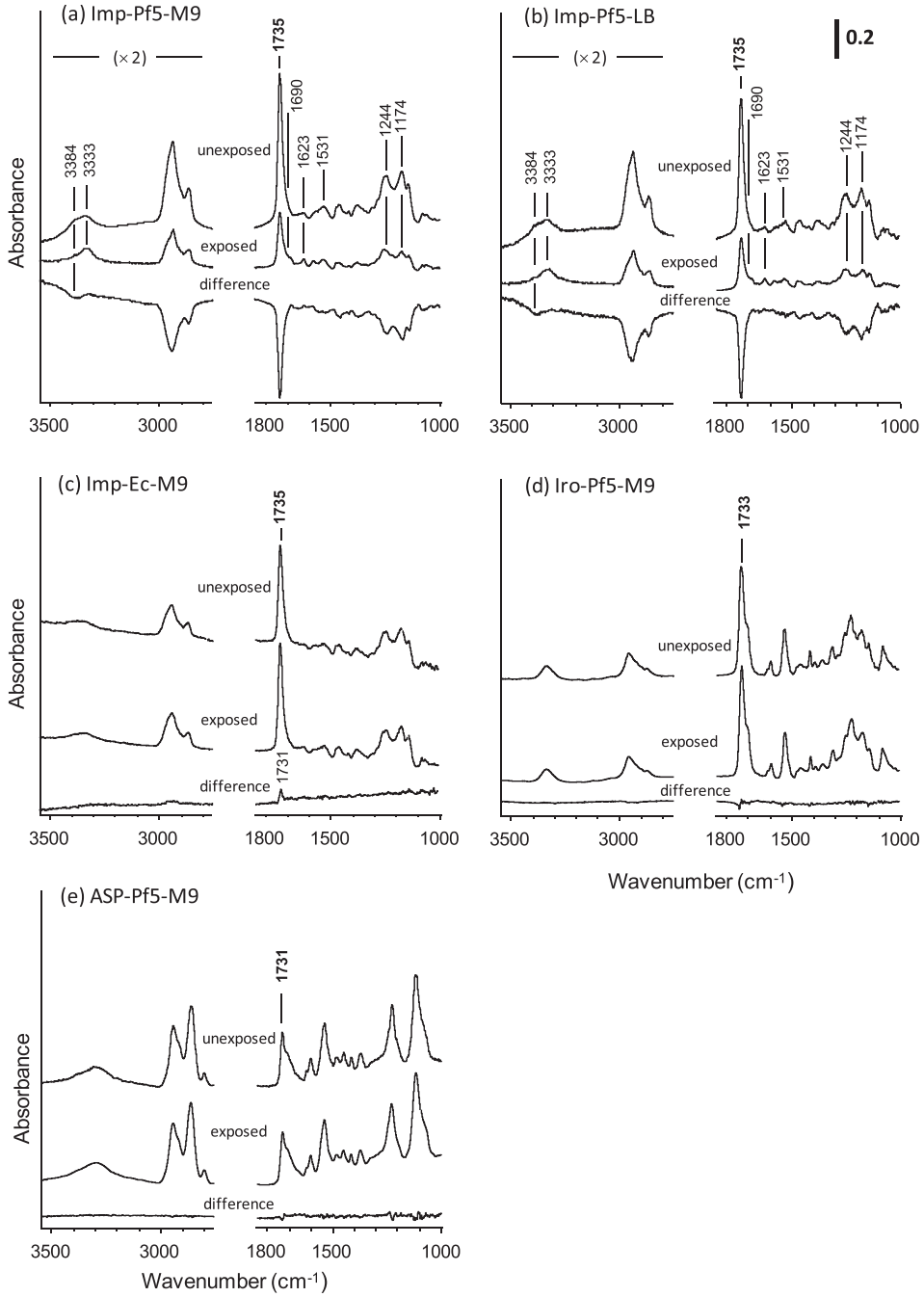

Fig. 7. Averaged μFTIR spectra from five coating assays used to quantitate coating material loss and identify chemical changes in remaining coating material following culture exposure: (a) Imp-Pf5-M9; (b) Imp-Pf5-LB; (c) Imp-Ec-M9; (d) Iro-Pf5-M9; (e) ASP-Pf5-M9 (abbreviations defined in text). Three spectra are shown for each assay and are labeled unexposed, exposed, or difference. The "unexposed" and "exposed" spectra are averaged μFTIR spectra collected at points within 300 μm of culture edges before and after coating exposure to the culture. The "difference" spectra are the "exposed" spectra minus the "unexposed" spectra. All spectra are shown on the same absorbance scale or adjusted by a factor of 2 where indicated.

largely absent from the spectra, further simplifying the bulk coating loss analysis.

Fig. 7a–e shows averaged μFTIR spectra from five coating assays used to quantitate coating material loss and identify chemical changes in remaining coating material following culture exposure. The five conditions assayed were (a) Impranil exposed to a Pf-5 culture grown on M9/citrate medium (Imp-Pf5-M9); (b) Impranil exposed to a Pf-5 culture grown on LB medium (Imp-Pf5-LB); (c) Impranil exposed to an *E. coli* culture grown on M9/citrate medium (Imp-Ec-M9); (d) Irogran exposed to a Pf-5 culture grown on M9/citrate (Iro-Pf5-M9); and (e) AS-P108 exposed to a PF-5 culture (ASP-Pf5-M9) grown on M9/citrate medium. Three spectra are shown for each assay and are labeled unexposed, exposed, or difference. The "unexposed" and "exposed" spectra are averaged μFTIR spectra collected at points within 300 μm of culture edges before and after coating exposure to the culture, with coordinates aligned relative to fiducial points on the coupons. The "difference" spectra are the "exposed" spectra minus the "unexposed" spectra. Thus, positive peaks in the difference spectra would represent chemical features present in the exposed spectra, but not the

unexposed spectra, and negative peaks would represent loss of chemical features following culture exposure. The carbonyl peaks used for quantifying coating material loss are indicated in Fig. 7 at frequencies between 1731 and 1735 cm^{-1}.

As expected from Fig. 6, the difference spectrum in Fig. 7a for the Imp-Pf5-M9 assay shows many negative peaks, including the carbonyl peak. The Imp-Pf5-LB assay also shows a comparable level of degradation while the Imp-Ec-M9, Iro-Pf5-M9, and ASP-Pf5-M9 difference spectra shown in Fig. 7c–e indicate no loss of coating material within experimental error. The Iro-Pf5-M9 and ASP-Pf5-M9 assays analyzed the Irogran and AS-P108 coating exposures to Pf5 under conditions that caused rapid Impranil degradation. The purpose of the Imp-Ec-M9 assay was to use *E. coli* colonies of the same size and preparation to the Pf-5 experiments and confirm that the presence of a culture does not remove the coating from the surface nor skew the IR analysis. For this assay, the difference spectrum in Fig. 7c does show a small positive carbonyl peak at 1731 cm^{-1} which may be a product from the culture. However, there is no indication of polymer degradation as indicated by the otherwise flat differential spectrum and lack of additional negative polymer bands. The differential carbonyl absorbance intensities from Fig. 7 are plotted in the bar graph in Fig. 8 for a simplified comparison of relative degradative coating material loss. The differential absorbances from the Imp-Pf5-M9 and Imp-Pf5-LB assays, which were the only ones to show appreciable coating degradation, correspond to thickness losses of 1.62 ± 0.08 and 1.5 ± 0.2 μm, respectively, for Impranil under the ambient conditions previously specified.

As mentioned earlier, the spectroscopic indications of coating loss revealed in Fig. 7a, b do not appear to be accompanied by corresponding increases in degradation product features. There are no new peaks or peak shifts observable on a scale equivalent to the other negative peaks in the difference spectra. Furthermore, with some small exceptions these difference spectra otherwise closely resemble inverses of the unexposed coating spectra, indicating that the difference spectra primarily reflect bulk loss of coating material with little indication of remaining degradation products. However, the Imp-Pf5-M9 and Imp-Pf5-LB spectra in Fig. 7a, b do still reveal some finer details of chemical changes in the remaining coating material following culture exposure. The frequencies of specific features to note are identified in Fig. 7. First, the decrease in the N–H stretch absorption intensity revealed in the difference spectrum occurs in the higher frequency range around 3384 cm^{-1}. The broader N–H stretch region in the "unexposed" Impranil spectra can be attributed to two different hydrogen bonding states of the urethane component with frequencies at 3384 and 3333 cm^{-1} (shown centered around 3341 cm^{-1} in Fig. 6). As the material degrades, the lower frequency state is favored. It is also possible there is some detection of residual degradation product at 3333 cm^{-1}. Likewise, a small shoulder that becomes observable on close inspection at 1690 cm^{-1} in the "exposed" spectra could be due to a relative increase in H-bonded carbonyl or a residual degradation product. It could also be due to a non-H-bonded urea carbonyl, (Coleman et al., 1997) which are common moieties in aqueous PU dispersions (including the H-bonded configuration) (Noble, 1997).

Further inspection of Fig. 7a, b also reveals changes in relative intensities for the pair of peaks at 1244/1174 and 1623/1531. In both cases, a reversal in relative intensity occurs with the higher frequency peaks showing slightly higher relative intensity with coating degradation. The 1244/1174 change was also noted for Fig. 6d. The peaks in this region are assigned to C–O stretches, but the urethane "amide III" mode is also expected at 1244 cm^{-1} (McCarthy et al., 1997). This indicates slightly higher urethane to ester ratios with degradation, also supported by the increased N–H to C=O peak area ratios noted for Fig. 6d. The 1623/1531 change is more difficult to understand. The 1623 cm^{-1} peak is assigned to a crystalline H-bonded urea carbonyl stretch (Coleman et al., 1997) while the 1531 cm^{-1} peak is assigned to "amide II" modes for both urethane and urea moieties. One possibility is that the urea groups remain in cross-linked, higher molecular weight degradation components that diffuse away less readily from the surface.

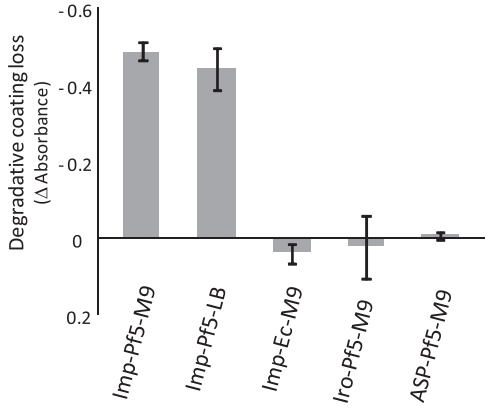

Fig. 8. Relative degradative coating loss as determined from changes in 1731–1735 cm^{-1} carbonyl absorbance intensities within 300 μm of culture edges relative to the same coating locations prior to culture exposure. The corresponding coating losses in μm for the Imp-Pf5-M9 and Imp-Pf5-LB assays are specified in the text.

4. Discussion

This work demonstrates a method using IR spectroscopy to quantify the biodegradation of PU films in proximity to an established PU degrading bacterium. Several strains of fungi and bacteria have been shown to degrade polyester PU through the activity of proteins classified as esterases or lipases (Nakajima-Kambe et al., 1999; Christenson et al., 2007; Loredo-Trevino et al., 2012). *Pseudomonas* strains are known to secrete enzymes that degrade PU polymers making them excellent test organism for new assays (Howard and Blake, 1999; Howard et al., 1999, 2001b; Shah et al., 2008, 2013; Mukherjee et al., 2011). Impranil was used as the primary test material since it could be compared to results from zone clearing assays. *E. coli*, which is not known to degrade PU, was used as a control bacterium to confirm that generic living cultures or media in contact with the coatings did not introduce artifacts into the analytical method. Unlike the zone clearing assays, other polymers could be tested using the coating assay like AS-P108 polyether PU and Irogran polyester PU coatings. The approach also allowed the interaction between bacterial cultures and polymer coatings to be monitored using small colonies (1 mm diameter) at early stages of culture development.

Acidity played a role in the growth and physiology of Pf-5 when grown on citrate, either in planktonic or plate cultures. Typically, over a 48 h period the pH around 1 mm colonies used in this assay could become >8.5. Interestingly, an alkaline shift in pH has been linked to increased esterase and lipase activity (Howard et al., 2012). The pH gradients established in the agar plates with a high concentration of cells (Fig. 4b) shows how the loading of cells can impact the agar gel and potentially complicate analysis from Impranil infused agar plate clearing assays. Large colonies consume nutrients more quickly than small colonies, generating more by-products per cm^2 of plate area, and thus cause larger and more rapid local pH changes than smaller colonies. The protocol used to decrease the number of cells applied in this assay allows for quicker

and a more straightforward analysis of degradation at early stages of growth but would ultimately suffer the same issues of overgrowth (colonies >>1 mm diameter) at longer durations, as discussed above.

The IR methodology demonstrated herein quantitatively identified a reduction in the film thickness and changes in the chemical composition consistent with qualitative observations of PU degradation in Impranil clearing experiments. The intensity of the carbonyl peak in the IR spectra of the PU coatings proved to be an excellent indicator of the effect a Pf5 culture had on the coating. Consistent with Impranil plate clearing experiments, radially collected spectra showed a spatially dependent change in the intensity of the carbonyl peak for both Imp-Pf5-M9 and Imp-Pf5-LB (Figs. 6–8). Furthermore, analysis of the interference fringes in the broad IR spectra presented in Fig. 6 revealed measurable changes in the coating thickness which supports the loss of coating material.

When PU degrades, the potential ester and urethane hydrolysis products would include alcohols and carboxylic acids. These would show –OH stretch bands, appearing near 3200–3400 cm^{-1} for the alcohol and over a broader region from 2500 to 3300 cm^{-1} for the carboxylic acid (Rodrigues da Silva et al., 2010). Carboxylic acids would also show carbonyl stretch absorptions shifted to about 1700–1725 cm^{-1} from the 1735 cm^{-1} ester carbonyl band, or 1550–1620 cm^{-1} in the carboxylate form. However, in the current studies, for reasons currently being explored, these metabolites were not detected in the spectra. One possible explanation for the absence of detected metabolites is that they were further metabolized by the bacteria. Alternatively, other work has shown that lipase and esterase breakdown of polyester PUs can produce low molecular weight metabolites such as diethylene glycol, trimethylpropane, and adipic acid (Nakajima-Kambe et al., 1997; Gautam et al., 2007). If these or similar low molecular weight metabolites were produced they could have diffused into the agar gel proximal to the culture during the 24hr exposure. The current work did not extend to a spectroscopic investigation into the composition of the agar gel.

As shown in Figs. 7 and 8, films of Impranil appear to be readily degraded under the same conditions used for the zone clearing assays (Fig. 1) but polymer films of AS-P108, a polyether PU, resisted degradation by Pf-5 (Fig. 7e), suggesting that the Pf-5 culture does not cause appreciable urethane breakdown within 24 h. Generally, polyester PUs are known to be more prone to biodegradation than polyether PUs, confirming that the urethane functionality is a more chemically robust linkage (Loredo-Trevino et al., 2012). This is consistent with Pf-5 primarily secreting esterases or lipases under these conditions. However, Irogran, another polyester PU, also resisted degradation over 24 h (Fig. 7d). More work will be required to fully understand the reasons for these differences but the current results signify that measures of Impranil degradation activity do not necessarily represent similar activity towards PU materials in general.

The increase in the NH to carbonyl peak ratios with Impranil degradation in Fig. 7a,b shows that the ester component is preferentially hydrolyzed and confirms that the primary mode of Impranil degradation was through breakdown of the ester component of the block co-polymer. The additional spectral details for Fig. 7a,b also indicated slightly higher ester versus urethane component loss from the Impranil and possibly some reorganization of remaining urethane and urea groups as degradation progressed. Based on analogy to previous work by Howard et al., two esterases (*pue*A and *pue*B) were considered the main candidates for facilitating this degradation, but it is possible that other esterases or lipases in Pf-5 may participate in polyurethane degradation (Howard et al., 2007). Recently, the term "polyurethanease" has been used (Stern and Howard, 2000; Howard et al., 2001a,b) for proteins with esterase activity that also hydrolyze Impranil. However, inconsistent reactivity with the urethane functionality and other PU formulations besides Impranil would make this term inaccurate.

When considering the generality of the spectroscopy described herein, it is important to consider its applicability and its limitations. The fact that Impranil degradation extended to coating regions beyond contact with the culture was fortunate in that it allowed spectral coating analysis without additional complication from culture contributions. The results suggest that diffusion of secreted compounds and proteins must have occurred during the coating assays. Spectroscopic analysis of a culture covered polymer coating could be complicated by the presence of the culture, but could be minimized by analyzing near the edge of the culture where the thickness is on the order of about three bacteria. Thus, unlike the zone clearing assays, this method is suitable for early culture development studies or experiments at the single-cell scale. While the current method is incapable of investigating the effects of thicker cultures on polymer coatings, a variation using cross-sectional techniques would allow for such experiments. Furthermore, cross-sectional techniques could enable analysis of a still-attached agar gel, offering a method to investigate the possible diffusion of metabolites away from the culture. As a final note, the direct culture – coating contact used in this assay should also be useful to analyze situations where membrane bound enzymes play a dominant role in microbial polymer degradation.

In summary, we report a plate-based assay using spectroscopic analysis of 1 mm colonies of *P. protegens* Pf-5 to determine if an early stage culture degrades PU coatings and films. In general, the assay was straightforward and amendable to any variation of agar growth support. While more work will be required to further resolve the full picture of the chemical changes occurring in the Impranil degradation, the results clearly demonstrate the ability to quantitatively compare polymer coating degradation using different culture/coating/media combinations. Colloidal Impranil was demonstrated as a suitable substrate for esterase activity assays in both solid coating and aqueous forms, however, as also shown, not all PU materials will be susceptible to rapid breakdown by these esterases which makes the indiscriminate use of Impranil for generic PU biodegradation questionable.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This study was supported by the Air Force Office of Scientific Research under award number 12RX14COR.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2014.09.005

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